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13. ABSTRACT *(Maximum 200*

The goal of this project was to produce a safe and effective molecularly cloned live virus vaccine for Venezuelan equine encephalitis virus (VEE). A panel of attenuating mutations was generated using biological selection techniques and *in vitro* mutagenesis targeted to regions conserved among alphaviruses. The full-length cDNA clone of the virulent Trinidad donkey strain of VEE was altered by site-directed mutagenesis to contain multiple attenuating mutations. Several multiple mutant clones were constructed, and progeny viruses, produced by transfection of cultured cells with RNA transcripts, were tested for virulence and immunogenicity in rodent models. The candidate vaccine, designated V3526, contains a deletion of the PE2 cleavage signal in conjunction with a second mutation at E1 position 253, from phe to ser. V3526 is avirulent in mice inoculated subcutaneously, intraperitoneally and intranasally, and is highly attenuated even when inoculated directly into the brain. Immunization with V3526 induces strong protection in mice against both parenteral and intranasal challenge with virulent VEE. Construction of attenuated mutants like V3526 may be possible for other alphaviruses, all of which contain similar PE2 cleavage signals.

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CONTAINS UNPUBLISHED RESULTS

INTRODUCTION

Experience in humans with the investigational live virus vaccine for VEE, TC-83, has shown that it is possible to produce solid protection against VEE safely by immunization with a live virus vaccine. TC-83-induced protection was demonstrated against both parenteral and aerosol challenge (Jahrling and Stephenson, 1984). However, TC-83 only produced this desired result in 65-70% of vaccinees. It gave mild to severe symptoms in about 15% of vaccinees, and did not produce an antibody response in another 20% (Edelman, 1986). The virulent Trinidad donkey strain of VEE (TRD) was passed in cultured cells over 80 times to produce the attenuated TC-83 strain, a procedure that led to accumulation of a small number of mutations that reduced virulence, perhaps as few as two (Kinney at al., 1993), as well as mutations that reduced the immunogenicity of the virus. In this case, the goal of generating multiple attenuating mutations while avoiding debilitating changes was only imperfectly realized.

The advent of recombinant DNA technology coupled with discoveries made using powerful genetic selection techniques with model alphaviruses (Olmsted et al., 1984) suggested that a new improved genetically engineered live virus vaccine for VEE could be produced. Attenuating mutations would be identified, evaluated and then combined in a single, multiply attenuated genome. To this end, a full-length cDNA clone of virulent TRD VEE, pV2000, was constructed from which infectious RNA genome replicas could be transcribed *in vitro* (Davis, et al., 1989; 1991). Baby hamster kidney (BHK) cells transfected with these RNAs gave rise to progeny virus whose genome sequence reflected that of the cDNA clone. Virus produced from

pV2000 gave a disease in rodent models and in horses that was indistinguishable from that produced by the parental TRD. Single site mutations, identified in a panel of attenuated mutants of TRD selected for the ability to rapidly penetrate BHK cells, were placed into pV2000 and shown to significantly reduce virulence in rodents. Molecularly cloned mutants that contained two or three such mutations were constructed, and were shown to be less virulent in sensitive rodent models (Davis, et al., 1991) and in horses (J.F. Smith, N. L. Davis, W. Laegreid, C. Bartz, G. Greenwald and R.E. Johnston, unpublished results).

The feasibility of this approach was demonstrated by these initial results. However, work with Sindbis virus suggested that improvements could be made in individual mutations (Schoepp and Johnston, 1993). Also, in theory, the constellation of changes chosen for the vaccine strain could be optimized. Three major areas of effort were outlined. First, the panel of attenuating mutations would be expanded, primarily by directed mutagenesis of regions conserved among alphaviruses, to give a large selection of changes that reduce virulence while maintaining the immunogenicity of the virus. Second, known mutations would be improved by comparing the attenuation afforded by deletions and by different amino acid substitutions at the same locus. Loci at which the majority of changes were lethal or attenuating would be preferred. Third, different combinations of mutations would be constructed and tested in animal models. The goal would be to choose mutations affecting different steps in virus replication, such that a single second site reversion could not negate the effect of more than one attenuating change. In an ideal combination, at least one constituent mutation would reduce infectivity for, and transmission by, the mosquito vector. The final candidate vaccine would be avirulent by a number of immunization routes in animal models, and would give solid protection against both

parenteral and aerosol challenge. Methods for broadening the protection of such a vaccine to other VEE subgroups would be explored. The successful application of this strategy to the problem of an improved VEE vaccine would serve as a model in the development of a new generation of safer, more effective genetically engineered vaccines for other viral pathogens, particularly other alphaviruses.

This report describes work that led to the identification, construction and characterization of a molecularly cloned vaccine candidate with the properties outlined above. Included are recombinant DNA strategies, tests of virulence and immunogenicity in rodent models, comparisons of pathogenesis phenotypes, and studies in cell culture.

RESULTS

A. Identification of Additional VEE Attenuating Loci

Several attenuating mutations were identified previously by sequence analysis of the glycoprotein genes of rapid penetration mutants of VEE, generation of full-length molecular clones containing these single nucleotide changes, and demonstration that viruses generated from such clones were attenuated in mice (Davis et al., 1991). Several approaches were taken to expand this panel to include the largest possible number of independent mutations for constructing a multiply attenuated vaccine candidate.

Further analysis of rapid penetration mutants

Three rapid penetration mutants shared a change from gln to arg at E2 position 81. One mutant was virulent, while the other two were attenuated. Testing of molecular clones

carrying E2 arg 81 showed that this mutation was attenuating in adult C57Bl/6 mice, and suggested that the virulent rapid penetration mutant carrying this change also carries a second mutation that gives it increased virulence without altering its rapid penetration phenotype. This has also been seen in the Sindbis virus-neonatal mouse system (Pence et al., 1990). Further work with the E2 arg 81 molecular clone demonstrated that reversion to the more virulent phenotype, possibly by the same second site mutation seen in the original panel, occurs during growth in tissue culture, since early harvests following transfection of baby hamster kidney (BHK) cells with the mutant RNA transcripts were attenuated, while later harvests were more virulent. Since some passage in cultured cells will be required in production of the live virus vaccine, it appeared that E2 arg 81 was too unstable to be included in a vaccine strain.

Analysis of mutations originally identified in TC-83

One of the sequence changes that distinguish the investigational VEE live virus vaccine (TC-83) from its virulent parent (Trinidad donkey strain VEE), an A for G substitution at nucleotide 3 of the 5' noncoding region, has been shown to reduce virulence in mice (Kinney et al, 1989; 1993). All three possible substitutions at this site were produced in the full-length VEE clone, V3000, shown to give viable virus, and subjected to several tests for their effect on virus virulence. For more detailed studies, the 516 nucleotide XbaI-RsrII 5'-terminal fragment was removed from each of these molecular clones, sequenced in its entirety and substituted for the homologous segment of V3000. Thus, within the sequence subjected to *in vitro* mutagenesis, the desired change at nt3 was the only difference between the mutant genome and the parent V3000. Both the original constructs and the sequenced clones were used to infect mice of different ages by different routes (Table 1)

TABLE 1
Attenuating mutations at nucleotide 3 of the 5' untranslated region

virus strain[a]	nucleotide at position 3	intraperitoneal: adult[b]	intraperitoneal: 2-week-old	virus strain[c]	subcutaneous: adult	intracerebral: adult
V3000	G	100 (8.2 ± 1.3)	100 (3)	V3000	100 (7 ± 2)	100 (5.2 ± 1)
U27E	U	87.5 (8.7 ± 1.2)	91 (3.9 ± 1.4)	V3045	100 (8 ± 1)	nd
U2811	C	0	82 (7.8 ± 1.4)	V3036	12.5 (8)	50 (6.8 ± 1)
U4011	A	12.5 (13)	64 (7.6 ± 1.7)	V3043	25 (10 ± 2.8)	62 (8.8 ± 1.3)

[a]Original molecularly cloned virus strains. [b]All inoculations were 10^3 pfu in CD-1 mice. [c]Molecular clones with sequenced replacements.

The substitution of either an A (as in TC-83) or a C at this locus significantly attenuated the virus, even following direct inoculation into the brain, while a change to U was much less attenuating. This attenuation locus may be an important component of a vaccine strain for two reasons. Changes at this site are predicted to affect the virus life cycle differently from mutations in the glycoprotein genes (See "Evaluation of Molecularly Cloned Viruses in Cultured Cells"), and these mutations have no effect on the antigenic structure of the virus particle.

Mutagenesis of regions conserved among alphaviruses

Several regions of the VEE genome show extensive homology with other alphaviruses (Ou, et al., 1983; Kinney et al., 1986; 1989). They have been conserved in these rapidly evolving genomes because they perform vital functions in virus replication that may involve interaction with elements of the host cell. Changes in these regions may have specific effects on the host range, tissue tropism or virulence of the virus.

1. The putative fusogenic region of E1. The portion of the E1 glycoprotein between amino acid residues 74 and 108, because of its conservation and lack of charged

residues, is proposed to be the fusogenic peptide involved in virus entry (Kinney et al., 1986; Garoff et al., 1980). Analysis of Sindbis virus mutants identified attenuating mutations at positions 72 and 75 (Polo and Johnston, 1990). Saturation mutagenesis was used to produce a panel of full-length clones carrying changes in this E1 region (Table 2).

TABLE 2
Mutations in the conserved region of the E1 glycoprotein gene

E1 codon	parental residue	mutant residue(s)		
80	val	ala		
81	phe	ile[a]	val	tyr
82	thr	ile	pro	
83	gly	trp[b]	ala	
84	val	phe		
85	tyr	asn		
87	phe	val		
89	trp	arg	gly	
90	gly	val	asp	
91	gly	ala		
92	ala	ser		

[a]Gave infectious RNA transcripts. [b]Produced a putative second-site revertant.

Sixteen of the 17 mutations were lethal for virus growth in BHK cells, as the RNA produced from clones containing these changes showed significantly reduced specific infectivity compared to the parental RNA transcripts. E1 ile 81, however, did give infectious RNA transcripts, and virus produced from this clone was avirulent for adult C57Bl/6 mice inoculated with 10^3 pfu intraperitoneally (ip.). Mice infected with this mutant were completely protected against challenge with 10^4 pfu of V3000. In addition, a putative second site revertant was isolated following transfection with the E1 trp 83 clone. This revertant also was avirulent and gave

protective immunity. The second site mutation has not been mapped. These results suggest the feasibility of this approach, in spite of the low tolerance for amino acid changes in this region.

2. The maturation cleavage signal between E3 and E2. The cleavage site used in the processing of PE2 to give the mature E2 glycoprotein is also conserved among alphaviruses, as well as other enveloped viruses (Schlesinger and Schlesinger, 1986; Hosaka et al., 1991). Mutations at the PE2 cleavage site that lead to incorporation of unprocessed PE2 into progeny virions are lethal in two alphaviruses, Semliki forest virus (Salminen, et al., 1992), and Sindbis strain AR339 (Heidner, et al., 1994), but can be accommodated in another Sindbis group alphavirus, S.A.AR86 (Russell, et al., 1989). To determine whether abrogation of PE2 processing is lethal for VEE, two different types of mutation were introduced into V3000 by site specific mutagenesis. One mutation deleted the entire four amino acid cleavage signal (E3 56-59, arg-arg-lys-arg). Alternatively, a panel of single nucleotide changes were introduced into the E3 59 codon, giving a series of amino acid substitutions for the final arg of the cleavage signal. These substitutions included leu, ile, thr, pro, asp, asn, phe, lys, met, ala, val, gln, tyr and glu. Full-length cDNA clones containing the deletion or any one of these substitutions produced nonviable RNA genomes, showing substantially reduced specific infectivities (Table 3). However, when chicken embryo fibroblasts (CEFs) were transfected with transcripts of the full-length clone carrying the cleavage signal deletion (pV3022), a delayed but characteristic cytopathic effect (CPE) was observed (in collaboration with J. Smith, USAMRIID). Culture supernatants harvested at 60 hr post-transfection contained infectious virus with a very small, irregular plaque phenotype. Sequence analysis of the E2 and E3 genes from viable viruses derived from pV3022 transfection showed only single amino acid changes from the sequence of

the original pV3022 RNA transcript (Table 3).

Full-length clones were engineered to contain either the lethal cleavage signal deletion plus the second change to ser at E1 position 253, or a lethal amino acid substitution at the PE2 cleavage site, [asp (GAC) for arg (AGA), requiring a double reversion (to CGC) to restore the parental arg], with or without the E1 ser 253 mutation. Analysis of these clones would test whether the E1 ser 253 mutation was responsible for resuscitating the cleavage site mutation in V3022 and whether the restorative ability of this mutation was limited to the deletion carried by V3022. As a control, a clone containing the E1 ser 253 mutation in the virulent V3000 background was produced. The viability of RNA genomes produced by clones containing only the E1 ser 253 mutation in conjunction with lethal PE2 cleavage signal mutations identified the change from phe to ser at E1 codon 253 as a second site suppressor of at least two types of PE2 cleavage site mutation. The viability of pV3040 transcripts showed that the E1 ser 253 mutation by itself does not affect the infectivity of V3000.

The elimination or mutation of the highly conserved PE2 cleavage signal resulted in the production of progeny VEE virions containing the unprocessed PE2 glycoprotein and lacking detectable mature E2, as demonstrated by RNA transfection or infection of BHK cells followed by polyacrylamide gel electrophoresis of purified ^{35}S-methionine labeled virions. This was the case whether or not the viability of the virions had been restored by a second site mutation.

The possibility that the infectivity for BHK cells shown by resuscitated cleavage defective mutants was generated by a very small subpopulation of virions with mature E2 on their surface was ruled out by comparing the specific infectivities (pfu/^{35}S methionine cpm) of

purified radiolabeled virions produced by transfection or infection of BHK cells (Table 3). The ratio of infectious particles (pfu) to total particles (cpm) was comparable for all of the genotypes, indicating that the PE2-containing particles are infectious. In addition, the resuscitating mutation in E1 restored normal levels of infectivity to both types of noncleaving mutant, although it was originally isolated in conjunction with the deletion of the PE2 cleavage signal.

TABLE 3
Properties of Molecularly Cloned PE2 Cleavage Site Mutants

Clone	Sequence at PE2 cleavage Site[a]	Residue at E1 253[a]	Viability of RNA Transcript[b]	Relative Specific Infectivity of Progeny Virus[c]
V3000	RKRR/S	Phe	+	1.0
V3022	----/S	Phe	-	0.0
V3038	RKRD/S	Phe	-	0.008
V3526	----/S	Ser	+	1.2
V3528	RKRD/S	Ser	+	1.2
V3040	RKRR/S	Ser	+	3.2

[a]Indicated mutations were placed in the full-length virulent clone, V3000, by replacement of mutagenized restriction fragments including either the E1 gene, the E2 gene or both. [b]*In vitro* RNA transcripts were quantitated by radiolabeling with ^{35}S-UTP and used with Lipofectin (BRL) to transfect BHK cell monolayers, which were then overlayed with agarose to produce RNA-initiated plaques. Viability was assessed by comparing plaque forming units (pfu)/counts per minute (cpm) to a V3000 transcript control. + indicates specific infectivity between 10% and 100% of that measured in parallel for a viable control transcript, - indicates specific infectivity < 1% of viable control. [c]BHK cell monolayers were either transfected with RNA transcripts (V3000, V3022, V3038, and V3040) or infected with transfection supernatants (V3000, V3526 and V3528) in the presence of ^{35}S-Met. Radiolabeled virions were purified and assayed for pfu and cpm. Values for pfu/cpm were calculated relative to V3000, set at 1.0, after correction for the Met content of E3. V3022, V3038 and V3040 were compared to V3000 in a single electroporation experiment. Values (relative to a V3000 control) for V3526 and V3528 represent an average of two separate infections.

Attenuation in animal models has been a consistent feature of PE2-containing alphavirus mutants (Russell, et al., 1989; Glasgow, et al., 1991; Heidner, et al., 1994). Results obtained with V3526 and V3528 (Table 4) extend this observation to PE2-containing mutants of VEE. Both V3526 and V3528 were avirulent, with no disease signs, when inoculated

subcutaneously (sc.) under conditions that give reproducible mortality with V3000. V3526 was also significantly attenuated when introduced directly into the brain at a dose that for V3000 routinely gives 100% mortality with an average survival time of 7.0 ± 0.0 days. In comparison with several other molecularly cloned attenuated viruses, both single and multiple mutants (Davis, et al., 1991), these PE2-containing mutants were the most highly attenuated in adult mice. The attenuation observed with V3040, containing the E1 ser 253 mutation alone, although unpredicted, was significant. The immunogenicity of all of these mutants was demonstrated by the resistance of immunized mice to intraperitoneal (ip.) challenge with a dose of V3000 (10^4 pfu) that gave 100% mortality with an average survival time of 5.5 ± 2.5 days in PBS-immunized controls.

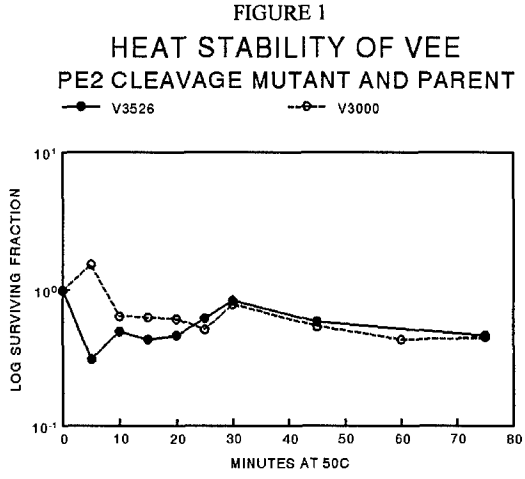
TABLE 4
Mouse Virulence Phenotypes of PE2-containing VEE Mutants

Clone	Route of inoculation	Deaths/Total (average survival time)
V3000	subcutaneous[a]	4/4 (8 .8 ± 0.75 days)
V3526	subcutaneous	0/8
V3526	intracerebral[b]	1/8 (8 days)[c]
V3528	subcutaneous	0/8
V3040	subcutaneous	1/8 (9 days)

[a] 10^3 pfu were inoculated sc. into the left rear footpad in a volume of 10µl. [b] 10^3 pfu were inoculated ic. into the left hemisphere near the midline in a volume of 10 µl. [c] In similar experiments with V3000, mortality following ic. inoculation of 10^3 pfu was 100% with 7.0 ± 0.0 days average survival time.

Initial attempts to purify PE2-containing viruses by centrifugation on potassium tartrate gradients were hampered by the apparent instability of these virus particles in high salt. This preliminary finding suggested that they may be generally less resistant to environmental

FIGURE 1



stress than their virulent parent. Therefore, V3000 and V3526 were tested in parallel for stability during incubation at 50C (Fig.1). The mutant and parent showed identical heat stability. The titers dropped only 50% after 75 minutes at the high temperature. Therefore, the structural alterations caused by the presence of PE2 instead of E2 in the virion must affect only some environmental conditions, eg. high salt, rather than reducing stability in general.

A new attenuating mutation in the E1 gene

Analysis of one of our molecularly cloned attenuated mutants suggested that a mutation in codon 272 of the E1 glycoprotein might be a new attenuating mutation (in collaboration with G. Ludwig and J. Smith, USAMRIID). The shuttle plasmid (see "Construction of Multiply Attenuated Mutants") was used to place the change from ala to thr at E1 position 272 into the pV3000 clone, and the replaced sequence was checked by direct RNA sequencing. Virus produced from this mutant clone carried a new site for N-linked glycosylation in E1 and gave only 11% mortality following either intracerebral or footpad inoculation of adult CD-1 mice. Therefore, our *in vitro* mutagenesis studies generated a fortuitous change in E1 that is attenuating.

B. Improvement of Known Attenuating Mutations

Small in-frame deletion at an attenuating locus

CONTAINS UNPUBLISHED RESULTS

Reversion of attenuating mutations to a more virulent phenotype is the most serious problem in the use of a live virus vaccine. Reasoning that attenuating deletion mutations will not be able to revert (although in theory they can be suppressed by second-site mutations), we tested deletions in a known attenuating locus, E2 codon 76. A substitution of lys for glu at this locus was the most attenuating mutation identified among the fast-penetrating mutants (Davis et al., 1991). A single codon deletion (3 nucleotides) at position 76, or a three codon deletion (positions 75, 76, 77) were independently introduced into the full-length clone using site-directed mutagenesis. Both of these deletions produced noninfectious RNA, as demonstrated by their low specific infectivity compared to parental V3000 transcripts. However, transfection of BHK cells with RNA containing the single codon deletion did give rise to progeny virus, which presumably contained both the deletion at E2 76 and a second resuscitating mutation. Two plaque isolates from this transfection supernatant were tested for virulence in adult female CD-1 mice. In a preliminary experiment, one isolate gave 30% mortality and an extended average survival time following inoculation of 10^3 pfu into the footpad, while the other gave 0% mortality. Inoculation in parallel with V3000 led to 100% mortality. It appears from these results that some loci will not accommodate a simple deletion mutation in a viable genome. Also, in this single case, second-site resuscitating mutation(s) were selected that gave rise to attenuated viruses, suggesting that inclusion of this type of double mutation in a vaccine strain may significantly reduce the probability of reversion.

Saturation mutagenesis of attenuating loci in the E2 glycoprotein

Work with another alphavirus, Sindbis virus, in a neonatal mouse model of pathogenesis suggested that the degree of attenuation produced by a single amino acid change

at a critical site in the virus glycoprotein depends on the particular amino acid that is substituted for the parental residue. Some attenuating loci show a large proportion of attenuating substitutions, while at other sites only one or a few substitutions are attenuating (Polo and Johnston, 1991; Schoepp and Johnston, 1993). Therefore, saturation mutagenesis of the attenuating loci at E2 position 76 and E2 position 209 of the VEE full-length clone was done to compare viability and attenuation afforded by all the possible amino acid substitutions at these two sites. The goal was to identify a site with a majority of lethal or attenuating substitutions, for which a double mutant attenuating codon can be designed such that two nucleotides would have to change to restore virulence. Eleven amino acid substitutions at E2 76 were made using the Kunkel mutagenesis procedure (Kunkel, 1985) in an M13 subclone of the VEE structural genes and, using the glycoprotein gene shuttle vector, eight of these mutations were placed into the full-length virulent VEE clone, V3000 (Table 5). Three of the mutations tested were lethal. Eight amino acid substitutions made at E2 position 209 and their effect on viability are also shown (Table 5).

Comparison of results with these two loci suggests that the E2 209 site may be more flexible, in that only two amino acid substitutions, *met* and *cys*, which are very similar chemically, gave a nonviable phenotype. At the E2 76 locus, however, three of the nine amino acids tested to date gave nonviable RNA genomes, and these residues represent very different chemical types. In addition, we showed previously that a single amino acid deletion at E2 codon 76 is also lethal. However, the final choice of site will rest not only on the proportion of lethal changes, but also on the proportion of attenuating changes, which remains to be determined. It will be of interest to determine the effect of different amino acid

substitutions at E2 codon 209 on the neutralization epitope that includes this locus. Work by others (Kinney et al., 1988 ; Johnson et al., 1990) and unpublished results obtained with V3014 (a double mutant containing E2 lys 209 and E1 thr 272, J.T. Roehrig, personal communication) indicate that a phe (in place of the parental ile) at E2 position 207 or a lys (in place of the parental glu) at E2 209 eliminate binding of an $E2^h$-specific monoclonal antibody. However, mutants containing the E2 lys 209 mutation are still able to elicit a protective immune response in mice and hamsters (Davis et al., 1991).

TABLE 5
Amino acid changes made at E2 positions 76 and 209

E2 amino acid 76[a]	Viability	E2 amino acid 209	Viability
glu (V3000)	+	thr (V3000)	+
ile	+	tyr	+
phe	-	pro	+
thr	-	thr	+
cys	ND[a]	cys	-
val	+	leu	+
leu	+	met	-
arg	+	ser	ND
ser	ND	his	ND
gln	ND	lys(V3032)	+
tyr	ND		
pro	-		
lys(V3010)	+		

[a]Not determined.

Saturation mutagenesis of E1 position 81

A change from phe to ile at codon 81 in the E1 glycoprotein produced a viable

mutant that was avirulent for adult mice. An additional 12 amino acids have been placed individually at this locus, using site-directed mutagenesis with pV3000 DNA as template and a degenerate primer, and the RNA transcripts produced from them have been tested for infectivity (Table 6).

TABLE 6
Full-length clones containing substitutions at E1 codon 81

clone designation	amino acid at E1 81	viability of RNA transcript
V3000	phe	+
cc3	gly	-
cc4	val	-
cc5	ser	+/-[a]
cc7	ala	-
cc12	thr	-
cc13	his	+/-
cc16	gln	-
cc21	leu	+/-
cc24	lys	-
cc25	arg	-
cc27	met	ND
cc32	ile	+[b]
cc36	tyr	-

[a]Specific infectivity of RNA (pfu/cpm ^{35}S-UTP incorporated was 7-20% of that for V3000. [b]Specific infectivity of RNA was 30% level of V3000.

Eight of the 12 clones tested gave noninfectious RNA transcripts. However, 3 of the clones gave RNAs with marginal specific infectivities; they may be infectious, or may be easily resuscitated by a second site change. The only clearly viable transcript came from the clone with the ile

substitution. These results underline the stringent requirement of this E1 region for a specific amino acid sequence. None of these mutants has been tested in mice.

C. Mutations Affecting Vector Infection and Transmission

Studies of growth in, and transmission by, Aedes taeniarhynchus have been done in collaboration with Dr. Mike Turell at USAMRIID. Infections were done by intrathoracic inoculation, and growth of virus was measured by plaque assay of pooled mosquito homogenates. Transmission was scored as positive when a susceptible hamster was exposed to an infected mosquito and subsequently showed clinical signs and/or survived a challenge with V3000.

Mutation at the PE2 cleavage site

As described above, viable revertants were isolated from tissue culture cells transfected with RNA carrying a lethal deletion of the PE2 cleavage signal. One of these double mutants, J9-1, contained the deletion coupled with a change from phe to ser at E1 position 253. A second, J2-8, carried a change from the parental leu to gln at E2 codon 243. Preliminary experiments with J2-8, which was attenuated in mice and hamsters, indicated that this double mutant grew poorly in mosquitoes. A full-length cDNA clone containing the deletion and resuscitation mutations in J2-8 was constructed, and virus produced from this clone gave reduced titers in the mosquito and was not efficiently transmitted to hamsters. Further studies were done with a molecularly cloned virus carrying the PE2 cleavage signal deletion plus the E1 ser 253 resuscitator, V3526. V3526 grew to one tenth the titer of V3000 in the mosquito, and only one out of 5 infected mosquitoes transmitted a hamster-avirulent virus on day 7. A molecularly

cloned PE2 noncleaving mutant carrying an asp substitution in the cleavage signal (E3 asp 59) plus the E2 gln 243 resuscitator, Z1a, showed very low titers in the mosquito until day 7, when titers were comparable with V3000. Hamster-virulent virus was transmitted by 3 out of 5 mosquitoes on day 7. This result is consistent with the generation of a hamster-virulent revertant during low level replication in the mosquito vector, although no sequence data is available on these mosquito-passed isolates. These results, coupled with the characterization of these PE2-containing viruses in cultured mosquito cells (see "Evaluation of Molecularly Cloned Mutants in Cultured Cells"), suggest that growth restriction in the insect vector as well as attenuation in the vertebrate host may be associated with these mutants.

A rapid penetration mutation near a neutralizing epitope

An escape mutant selected for resistance to neutralization by monoclonal antibody 1A3B-7 was found to have a single amino acid substitution at E2 position 207 (Johnson et al., 1990). This mutant grew to lower titers in an *Aedes albopictus* cell line than its parent, and was restricted in its ability to infect and disseminate from the midgut of *A. aegypti* mosquitoes following oral infection (Woodward, et al., 1991). Since one of the molecularly cloned attenuated mutants contained a single change at E2 position 209, it was tested for its ability to grow in mosquitoes following intrathoracic inoculation. Four of 5 infected mosquitoes were negative for virus on day 2, but by days 5 and 7, 4 of 5 mosquitoes showed titers as high as V3000. Three of 5 mosquitoes transmitted hamster-avirulent virus on day 7. These results may indicate that reversion during low level replication in the mosquito overcame the block to growth, but did not result in a more hamster-virulent phenotype. This locus may be a useful target for a double nucleotide change or a single codon deletion to give a less revertible mosquito

negative mutation.

A deletion mutation in the C-terminus of nsP3

The nonstructural protein, nsP3, is the least conserved among the alphaviruses, at both the nucleotide and amino acid level (Kinney et al., 1989). However, deletions in the C-terminal region, while having no effect on the viability of the mutant in cultured vertebrate cells, did decrease the efficiency of growth in cultured insect cells (La Starza et al., 1990). A viable deletion mutant of VEE, V1000, has been mapped to the C-terminus of nsP3 (Davis et al., 1989). V1000 grows as well as V3000 in three types of vertebrate cells and is as virulent in rodents. V1000 also grew as well as V3000 in mosquitoes inoculated intrathoracically, and was as efficiently transmitted to hamsters. It remains to be seen whether this mutant can infect and be transmitted by orally infected mosquitoes.

D. Construction of Multiply Attenuated Mutants

The strategy in designing a new live attenuated VEE vaccine strain with a low probability of reversion is to combine mutations that specifically attenuate virulence in a single cDNA clone from which infectious RNA transcripts can be derived. Previous results suggested that molecularly cloned viruses with two or three independently attenuating mutations were less virulent in a sensitive animal model (mice less than 2 weeks old) than viruses with only one of the component mutations, and that a strain carrying three such mutations could form the basis for a safe and effective live vaccine (Davis et al., 1991). However, the original triple mutant, V3507, grew to somewhat lower titers than V3000 on BHK cells. V3507 contained three mutations in the E2 glycoprotein gene which all were identified originally in rapid penetration

mutants (Johnston and Smith, 1988). It is possible that the combination of these three attenuating mutations, which all may affect the same step in virus replication, significantly reduced the efficiency of that step and restricted virus growth. Therefore, additional triple mutants were constructed to determine whether this was the case. First, a shuttle vector was constructed to allow the replacement of the E2 and E1 genes individually into pV3000.

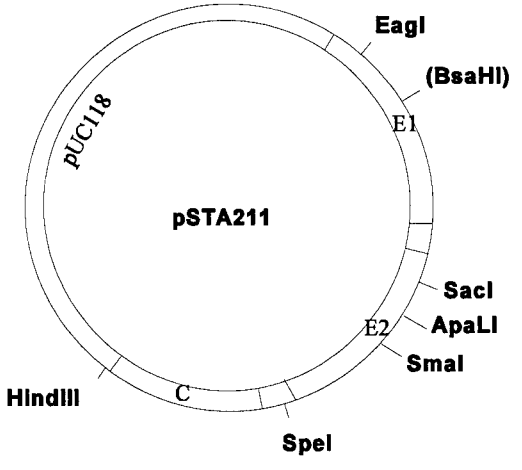
Construction of a shuttle vector for combining mutant E2 and E1 glycoprotein genes

A shuttle plasmid containing the structural genes of VEE was made such that unique restriction sites flank the E2 and E1 glycoprotein genes (Figure 2). First, a DNA fragment of about 4 kbp [HindIII, nt 7290, to NotI, downstream of the poly(A) tract] was removed from pV3000 and placed into the multiple cloning site of pUC118 (Viera and Messing, 1987). Next, a BsaHI site in the subcloned E1 gene (nt 10,848) was removed by exchanging the SacI-EagI fragment (nt 9492 to nt 10,890) for one in which the BsaHI site had been altered by site-directed mutagenesis without altering the amino acid sequence. Finally, the SmaI site (nt 9072), originally made nonfunctional by a site-directed silent mutation in pV3000, was reinstated in the subclone by exchanging the SpeI-ApaLI fragment (nt 8389 to nt 9220) of the subclone with the analogous fragment from the original cloned sequence of the Trinidad donkey strain of VEE, pV2000 (Davis et al., 1991). To test the authenticity of the resulting sequence, the DNA fragment flanked by the unique AflII (nt 8054) and SacII (nt 11,199) sites in the shuttle plasmid was used to replace the analogous region in the full-length pV3000 clone. *In vitro* RNA transcripts from this clone were infectious, and the sequence between the SacI and EagI sites (the only part of the subclone subjected to site-directed mutagenesis) was determined by direct RNA sequence analysis. The predicted amino acid sequence was identical to that of pV3000. Finally,

virus produced by transfection of BHK cells with these transcripts gave 100% mortality in CD-1 mice following injection of 10^3 pfu into the footpad with an average survival time identical to that for the V3000 control.

The shuttle plasmid allows the combination of individual mutations in the E2 and E1 genes with mutations in the 5'-noncoding region. Each gene replacement can be monitored with a restriction enzyme, either SmaI or BsaHI. Also, exchanged sequences that originate from mutagenized M13 subclones, which may contain unintentional changes, will be relatively small and can be checked easily by direct RNA sequencing.

FIGURE 2



Mutations in the E2 and E1 genes

Two triple mutant clones were constructed, both of which contained attenuating mutations at E2 positions 76 and 209 (both glu to lys), with an additional mutation in E1, either E1 thr 272 (V3519), or E1 ile 81 (V3520). Both constructs gave infectious RNA and reasonable titers on BHK cells.

CONTAINS UNPUBLISHED RESULTS

Mutations in the glycoprotein genes and the 5'-noncoding region

cDNA clones containing two E2 mutations, E2 lys 76 and E2 lys 209, in conjunction with an attenuating C at nt 3 of the 5' noncoding region gave noninfectious transcripts. To determine whether a different attenuating change at nt 3 would give viable transcripts when combined with E2 mutations, two additional triple mutant clones were constructed. The parental sequence between the XbaI restriction site (just upstream of the T7 promoter) and the RsrII restriction site (nt 516) in the two double mutant clones was replaced by the analogous fragment containing an A rather than the parental G at nt 3 of the 5' noncoding region. The entire replaced region was checked by direct RNA sequencing. This A for G substitution at nt 3 attenuated virulence both in adult and two-week-old CD-1 mice (Table 1). Kinney and coworkers also showed that this is an attenuating mutation in their work with TC-83 (1993). Both the mutant clone containing E2 lys 76, E2 lys 209 and nt3 A V3522) and the mutant containing E2 lys 209, E1 ser 272 and nt3 A (V3524)gave infectious RNA transcripts. However, V3522 gave pinpoint plaques on BHK cells, which raises the possibility that the nt3 C-containing triple mutants described above as nonviable was really viable, but gave plaques too small to be detectable in our assay. This type of multiple mutant may block two or more steps in pathogenesis to give a very low frequency of reversion to a virulent phenotype. The theoretical reversion rate at nt3 is high as only 2 of the possible 4 nucleotides at this position are attenuating (Table 1), and therefore there is a 50% chance that a change at this position would give a nonattenuating nucleotide. This factor must be weighed carefully before including a nt3 mutation in a vaccine candidate. In fact, this may be an important contributor to the reactogenicity of the TC-83 vaccine.

E. Evaluation of Molecularly Cloned Mutants in Rodent Models

Extensive testing has been done with single attenuated mutants in rodent models, both in mice, which show the same disease course and fatal encephalitis found in VEE-infected humans and equines, and in hamsters, which are highly sensitive to the lymphotrophic phase of viral infection. Initial testing of multiple mutants supported the hypothesis that combination of more than one attenuating mutation in a single virus strain would increase the level of attenuation, even in a very sensitive animal model (Davis, et al., 1991). However, it was clear that different combinations gave different virulence phenotypes and different levels of immunogenicity, and that each mutant constellation would have to be tested thoroughly in these models. These data are presented in this section.

Effects of different mouse strain and route of inoculation

V3000 and three single attenuated mutants were used to infect CD-1 mice (outbred) by subcutaneous (sc). inoculation of the rear footpad (fp.), or C57Bl/6 mice (inbred) by intraperitoneal, ip. inoculation. V3000 gave 100% mortality in both cases. Two of the mutants, E2 lys 76 and E2 lys 209, were completely avirulent in both cases. One mutant, however, E2 lys 120, was avirulent in the C57Bl/6 mice, but gave 78% mortality in the CD-1 mice. This higher mortality for the E2 120 mutant was also seen in one-week-old and two-week-old mice (Davis, et al., 1991). These results suggest that use of different mouse models may reveal quantitative or qualitative differences among attenuated mutants.

Measurements of mortality and average survival times (AST) were made following (fp.) and intracerebral (ic.) inoculation of CD-1 mice to compare attenuated mutants with respect to neuroinvasiveness and neurovirulence (Table 7).

TABLE 7
Per Cent Mortality and Average Survival Time of Adult CD-1 Mice Infected by Two Different Routes

	V3000		V3010 (E2 lys 76)		V3012 (E2 lys 120)		V3014 (E2 lys 209, E1 thr 272)	
	ic.	fp.	ic.	fp.	ic.	fp.	ic.	fp.
% Mortality	100	100	22	0	100	78	100	0
AST (days)	4.7 ± 0.7	5.4 ± 0.5	7.5 ± 0.7		6.0 ± 0.5	9.1 ± 0.7	7.7 ± 0.5	

In CD-1 mice, V3014 was clearly blocked at invasion of the central nervous system, while V3010 was inefficient at causing disease even when introduced directly into the brain. One strategy for producing a stable multiply attenuated vaccine strain is to combine mutations that block the disease process at different stages. The comparison of these two different routes of inoculation is a first step in distinguishing the blocks produced by individual attenuating mutations.

Testing of multiple mutants for attenuation and protection in animal models

1. Glycoprotein gene mutations. One class of multiple mutants contains three mutations in the glycoprotein genes. Two such mutants have been tested in rodent models, V3519 (E2 lys 76, E2 lys 209, E1 thr 272) and V3520 (E2 lys 76, E2 lys 209, E1 ile 81). Both triple glycoprotein mutants were avirulent when inoculated sc. (left rear footpad) into 5 week-old female CD-1 mice at a dose of 10^3 pfu and gave solid protection against an ip. challenge with 10^4 pfu of V3000 (Table 8). V3520 was also avirulent ic. at a dose of 10^3 pfu, while V3519 killed 1 mouse of 6. All of the survivors of ic. infection were also protected against V3000 challenge. V3519 and V3520 have now been tested for virulence and protection in C57Bl/6 mice and hamsters by our collaborators at Ft. Detrick (Table 9). These animals were inoculated sc. (back

of the neck) with a dose of 10^4 to 10^5 pfu and challenged with 10^5 pfu of V3000 either ip. or by aerosol. Both V3519 and V3520 were avirulent in C57Bl/6 mice, and V3520 killed only 1/20 hamsters with an extended survival time. However, in this experiment, neither strain gave complete protection of mice or hamsters against ip. or aerosol challenge with virulent V3000, consistent with the fact that neither elicited a serum neutralization titer over 1:36 (80% plaque reduction neutralization titer).

2. Glycoprotein gene mutations coupled with a 5'-noncoding region change. Two additional triple mutants were constructed containing an attenuating A at nucleotide position 3 of the 5'-untranslated region in combination with either E2 76 and E2 209 (V3522) or E2 209 and E1 272 (V3524). Initial experiments in 5 week-old female CD-1 mice showed that both of these mutants were avirulent when inoculated sc. (left rear footpad) at a dose of 10^3 pfu, and gave complete protection against an ip. challenge with 10^4 pfu of virulent V3000 (Table 8). V3522 was also avirulent and protective by the intracerebral route (ic.), while V3524 gave 100% mortality ic. Further studies with these mutants, and a comparison with viruses containing the single component mutations, were conducted at Ft. Detrick in C57Bl/6 mice and hamsters (Table 9). Both mutants were avirulent in C57Bl/6 mice following sc. inoculation (back of the neck), but only V3524 gave complete protection against aerosol and ip. challenge with V3000. In hamsters, V3522 was avirulent, while V3524 gave 1 death out of 20 animals. Both protected against ip. but not aerosol challenge in hamsters.

It is not clear at this time why triple mutants that afforded complete protection against V3000 challenge in CD-1 mice did not reproducibly protect C57Bl/6 mice. It is possible that the mouse strain, the route of immunization (footpad vs. back of the neck) and/or the amount

TABLE 8
Attenuation and Induction of Protective Immunity in Outbred Female CD-1 Mice

virus strain	attenuating mutations	route of immunization	survival (survivors/total)	challenge[b] (survivors/total)
V3000		ic.	0/3	
		fp.[a]	0/3	
V3519	E2 lys 76 E2 lys 209 E1 thr 272	ic.	5/6	5/5
		fp.	6/6	6/6
V3520	E2 lys 76 E2 lys 209 E1 ile 81	ic.	7/7	6/6
		fp.	6/6	6/6
V3522	E2 lys 76 E2 lys 209 nt3 A	ic.	4/4	4/4
		fp.	4/4	4/4
V3524	E2 lys 209 E1 thr 272 nt3 A	ic.	0/4	
		fp.	4/4	4/4

[a]Inoculations were sc. into the left rear footpad at a dose of 10^3 pfu. [b]Challenge was ip. at a dose of 10^4 pfu of V3000.

of V3000 challenge virus influenced the outcome. The survival of 2 of the V3000 inoculated mice in the C57Bl/6 experiment (Table 9) may indicate that infection by sc. inoculation in the back of the neck is less efficient than footpad injection, which has never given a survivor of V3000 at this dose. These inconsistent results do not establish whether or not these triple mutants are sufficiently immunogenic, and further direct comparisons in rodent models are needed. The greater sensitivity of the hamster to the disease caused by V3520 and V3524 may reflect the fact that these mutants are able to carry out at least the beginning of the lymphotropic phase, which may be enough to compromise the intestinal epithelium of some of the hamsters (Gleiser et al., 1962).

TABLE 9
Attenuation and Induction of Protective Immunity in Rodents[a]

virus strain	attenuating mutations	C57Bl/6 mice			Hamsters		
		immunization[b]	challenge		immunization[b]	challenge	
			ip.	aerosol		ip.	aerosol
V3000		2/10		2/2	0/10		
V3010	E2 lys 76	10/10		7/10	7/10		6/7
V3032	E2 lys 209	10/10		10/10	2/10		1/1
V3034	E2 thr 272	10/10		10/10	2/10		2/2
V3042	E1 ile 81	10/10		10/10	2/10		1/1
V3040	E1 ser 253	10/10		10/10	0/10		
V3519	E2 lys 76 E2 lys 209 E1 thr 272	20/20	6/10	2/10	20/20	4/10	0/10
V3520	E2 lys 76 E2 lys 209 E1 ile 81	20/20	3/9	9/10	19/20	4/7	3/10
V3522	E2 lys 76 E2 lys 209 nt3 A	20/20	5/10	3/10	20/20	9/9	7/10
V3524	E2 lys 209 E1 thr 272 nt3 A	20/20	10/10	10/10	19/20	9/9	9/10
V3526	RKRR del E1 ser 253	20/20	10/10	10/10	ND[c]		
V3528	E3 asp 59 E1 ser 253	20/20	10/10	10/10	ND		
saline		20/20	0/10	0/10	20/20	0/10	0/10

[a]Survivors/total. [b]Inoculations were sc. into the back of the neck at a dose of 10^4 pfu to 10^5 pfu. [c]Not done.

3. Further animal studies with PE2-containing mutants. Included in the experiments at Ft. Detrick were the resuscitated PE2-containing viruses, V3526 and V3528, and the E1 ser 253 resuscitating mutation in the V3000 background, V3040 (Table 9). These mutants were completely avirulent in C57Bl/6 mice and, in the case of the PE2-containing viruses, were

fully protective against V3000 challenge by either the ip. or aerosol route. V3040 protected completely against aerosol challenge in the mice, but was virulent for the hamsters. This suggests that the initial phase of infection, to which the hamster succumbs, is not blocked by the resuscitation mutation in the V3000 background.

Relative to several other molecularly cloned attenuated viruses, including both single and multiple mutants (Davis et al., 1991), these PE2-containing mutants appeared to be the most highly attenuated in adult mice(Tables 4 and 9). Initial comparison of several molecularly cloned vaccine candidates in C57Bl/6 mice at USAMRIID showed that V3526 infection, in addition to being completely innocuous in mice, also induced very high levels of anti-VEE serum IgG. In that test, V3526 gave higher geometric mean titers even than less attenuated single-site mutants. In a subsequent experiment in CD-1 mice, anti-VEE antibody production was compared for mice that were inoculated sc. in the left rear footpad with 10^3 pfu of either V3526 or V3014 (E2lys209, E1thr272), and boosted at three weeks with 10^5 pfu of the same virus. As observed previously, neither virus caused clinical signs in the mice. Geometric mean titers were identical between the two groups of mice after the first inoculation (17,954), and very similar after the booster inoculation (35,900 for V3526 and 31,989 for V3014). This result indicates that both of these two attenuated VEE strains induce a strong anti-VEE immune response, and that V3526 is not characterized in CD-1 mice by exceptional immunogenicity.

A possible basis for the strong attenuation and immunogenicity of V3526 may be seen in a study of the spread of V3526 in the mouse following sc. inoculation of 10^3 pfu in the left rear footpad. At various times post-inoculation two mice were sacrificed and several organs were harvested for virus titration. The draining popliteal lymph node was the only site of

significant virus replication, which was already well under way by 6 hrs, although in some animals virus also was found in the contralateral lymph node and Peyer's patches. Very low serum viremia was evident, and no virus was recovered in the brain at any time point. In a second study, concerned mainly with lymphoid spread during the first 24 hrs, V3526 was found in the draining popliteal lymph node and other nodes connected by the lymphatic system. Virus was also detected in the mandibular lymph nodes. This ability to seed several lymph nodes while sparing other tissues may explain the immunogenicity and strong attenuation of this mutant.

4. PE2-containing mutants with an additional attenuating mutation. Full-length cDNA clones were constructed containing a PE2 cleavage site mutation and E1 ser 253 resuscitator combined with one additional attenuating mutation. Our first attempt was a multiple mutant containing the PE2 cleavage site deletion, the resuscitator at E1 253 and the attenuating lys at E2 209. Several independent isolates of this clone failed to give infectious RNA transcripts, indicating that this combination is lethal. In subsequent constructs we added an attenuating change from the parental G to A at nucleotide 3 of the 5'-untranslated region, or the attenuating change E1 ile 81 to the PE2 cleavage site deletion and E1 ser 253 resuscitator. *In vitro* RNA transcripts from these clones were viable. The mutant containing the nt3 A mutation produced nearly normal sized plaques on BHK cells, while the mutant with the E1 ile 81 change produced extremely small plaques. The sequence of the region of E1 that was mutagenized to insert the E1 ile 81 mutation was confirmed by direct RNA sequencing. Virus stocks were produced by transfection of BHK cells, but no further testing of these viruses has been done.

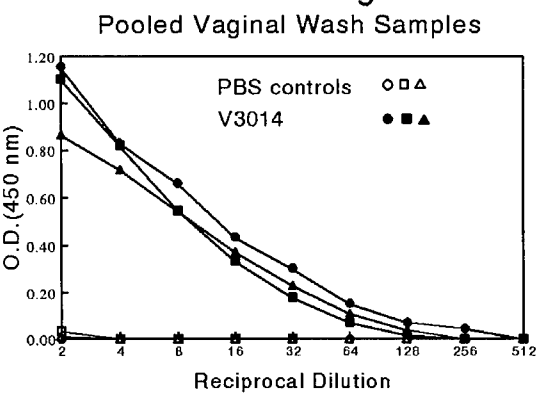
Protection against intranasal challenge with virulent V3000

VEE, unlike many other alphaviruses, can be spread by the aerosol route (Jahrling

and Stephenson, 1984). Therefore, the ability to protect against aerosol or intranasal infection would be an important property of a vaccine candidate. TC-83 protects against aerosol and intranasal challenge with virulent VEE (Jahrling and Stephenson, 1984; Ryzhikov et al., 1991). Subcutaneous footpad inoculation with 10^3 pfu of the molecularly cloned attenuated double mutant V3014 (E2 lys 209, E1 thr 272) afforded complete protection against intranasal (in.) challenge with 10^4 pfu of V3000. All eight adult female CD-1 mice immunized with V3014 survived intranasal challenge with 10^4 pfu of V3000 at three weeks post-immunization, while none of the 6 mice mock-immunized with diluent survived.

When the footpad, draining popliteal lymph node, spleen, olfactory neuroepithelium, olfactory bulb, brain and serum of challenged animals were assayed for the presence of viable virus, none was found at any of several time points between 6 and 24 hr post-challenge. It was possible that the high levels of anti-VEE serum antibody in these mice at the time of challenge may have masked a low level of replication of challenge virus. Therefore, *in situ* hybridization (as described in Grieder et al., 1995) with a VEE-specific riboprobe was used to assay for challenge virus replication. Tissues from nasal neuroepithelium, olfactory bulb and other CNS structures were assayed at 24 and 72 hrs after in. challenge with V3000. Tissues from V3000-challenged, mock-vaccinated mice showed large amounts of newly synthesized V3000 RNA, coupled with extensive pathology throughout the full thickness of the olfactory neuroepithelium and invasion of the CNS. However, in V3014-vaccinated mice no VEE RNA was detected. These data strongly suggested that the apparently complete protection against virulent in. challenge was mediated at the level of the nasal mucosa itself (Charles et al., 1997).

FIGURE 3



Anti-viral IgA has been implicated in protection of the nasal mucosa of mice against invasion by influenza virus and Sendai virus (Mazanec et al., 1987; Renegar and Small, 1991). Therefore, V3014-inoculated mice were assayed for VEE-specific mucosal IgA. Pooled and concentrated vaginal washes taken at 10, 14 and 21 days post immunization were used as the source of antibody in an ELISA with purified VEE virus antigen and goat anti-mouse IgA second antibody (Fig. 3). Significant levels of VEE-specific IgA were measured relative to unvaccinated controls. Delineating the mechanism by which a subcutaneous inoculation of attenuated VEE induces mucosal immunity may broaden our understanding of the mucosal immune system and the interaction between attenuated VEE vaccine strains and the host.

It appeared likely that if an attenuated virus could be introduced safely onto a mucosal surface, it could very efficiently induce a mucosal immune response. The resuscitated PE2 cleavage signal deletion mutant, V3526, was avirulent in CD-1 mice inoculated in. with either 10^4 or 10^5 pfu (Table 10). Subcutaneous and ic. inoculations were done as controls, and these routes also gave 0% mortality, except for the high dose ic. which gave one death in six mice. When these mice were challenged in. with 10^4 pfu of virulent V3000, they all survived with no signs of VEE-induced disease. Unimmunized control mice all succumbed to V3000 challenge with an average survival time of 7.25 days. Another attenuated mutant, V3014, gave 33% mortality (2/6) following in. inoculation with 10^3 pfu, and 2/4 immunized mice succumbed

to in. challenge with V3000 on day 8 post-challenge. Therefore, V3526 appears to be highly attenuated by the in. route, but is still able to establish an immunizing infection, while V3014 does not efficiently infect by the in. route, and when it does infect is likely to produce disease. Comparisons of the in. and sc. routes with additional multiple mutants followed by in. and/or aerosol challenge will give information concerning the preferred virus genotype and route for induction of mucosal immunity.

Serum and saliva samples were collected from some of the mice inoculated in. with V3526 three weeks post-inoculation and tested for VEE-specific IgG (sera) or IgA (sera and saliva). Serum IgG ELISA titers ranged from 1000 to 4000 (reciprocal of dilution required to give $O.D._{450} > 0.2$, measured against gradient purified V3000). All but one of the mice tested had high serum IgA titers (2000-4000), but no VEE-specific IgA could be detected in the saliva.

TABLE 10
Immunization with V3526 and Intranasal Challenge with V3000

Route of Immunization	Dose (pfu)	Immunization Deaths/Total (survival time)	Challenge Deaths/Total[a]
intranasal	10^4	0/6	0/6
	10^5	0/6	0/6
intracerebral	10^3	0/6	0/6
	10^5	1/6 (7 days)	0/5
subcutaneous	10^5	0/6	0/6

[a]For PBS-immunized controls challenged in. with V3000, deaths/total = 4/4 with an average survival time of 7.5 ± 0.75 days.

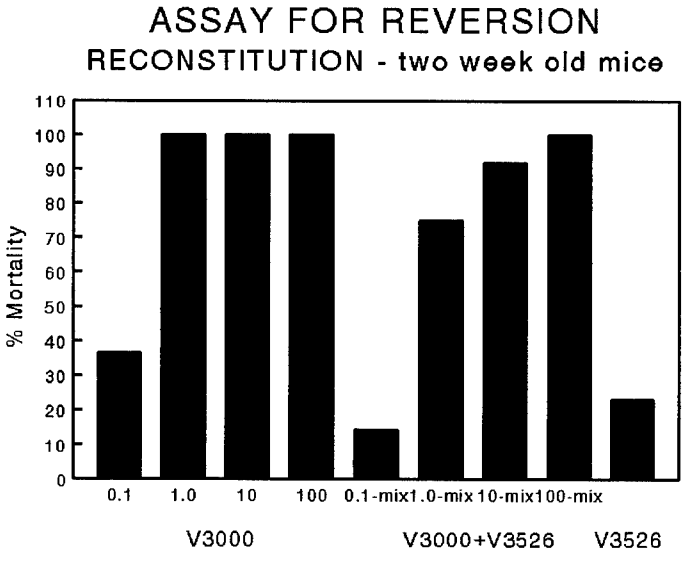
Therefore, although these mice were all resistant to a high dose in. challenge with V3000, and most had high levels of circulatory IgA prior to challenge, the levels of secretory IgA in saliva

were below our level of detection. In subsequent experiments, vaginal wash samples were shown to be a rich source of VEE-specific IgA antibody, but such samples were not taken in this experiment.

Reversion of molecularly cloned mutants in the animal host

The informed design of a safe and effective molecularly cloned VEE vaccine is based on the premise that combining multiple independently attenuating mutations will lower the rate of reversion to a virulent phenotype in the vaccinee. In very sensitive animal models we showed previously that multiply attenuated mutants gave lower mortality than single mutants (Davis et al., 1991), but direct measurement of reversion *in vivo* is difficult. One approach would be to biologically clone viruses from tissues of mice infected with vaccine candidates, and test each virus clone for virulence phenotype in naive mice. An alternative to this time-consuming protocol is to design a test in which virus-containing samples that may include a low proportion of virulent revertants, eg. tissue homogenates from vaccinated mice, are inoculated into animals and scored for morbidity and mortality. The fact that only a single plaque forming unit of virulent VEE is lethal for mice makes this type of test feasible. A preliminary test of such an assay was to mix highly attenuated V3526 with varying amounts of virulent V3000. Adult CD-1 mice

FIGURE 4



were inoculated ip. with 10^5 pfu of V3526 mixed with varying amounts of V3000 (from 0.1 pfu to 1000 pfu). Inocula were back-titrated to measure exact doses. In controls receiving only V3000, all inoculated mice died at a dose of 1 pfu or above. However, mice receiving the mixed viruses showed 16% mortality at 1 V3000 pfu, 0% at 10 pfu, 30% at 100 pfu and 16% at 1000 pfu. 10^5 pfu of V3526 alone gave 0% mortality. This first experiment showed that in the adult mouse, the presence of an otherwise lethal amount of V3000 when mixed with 10^5 pfu of V3526 did not consistently result in death. Therefore, the same experiment was carried out in more sensitive two week old CD-1 mice (Fig. 4). In this test, even 0.1 pfu of V3000 alone gave 35% mortality, with all higher doses resulting in 100% mortality. 10^5 pfu of V3526 alone gave 25% mortality. However, mixing only 1 pfu of V3000 with 10^5 pfu of V3526 resulted in 75% mortality, within the range that would be predicted for a 1 pfu dose of V3000 alone. Higher amounts of V3000 produced 95% and100% mortality. Thus, in two week old mice, a reversion frequency of 10^{-5} could be detected. When this protocol is applied in the actual assay to tissues from infected mice, the presence of cytokines may interfere with the results. Further testing is required to determine whether such interference exists, and what remedies can be applied. Eventually, ip. inoculation of serial dilutions of clarified tissue homogenates from vaccinated mice into two week old CD-1 mice will provide a sensitive screen for the rate of reversion to a virulent phenotype.

F. Evaluation of Molecularly Cloned Mutants in Cultured Cells

Comparative growth studies in various types of cultured cells have been used in two contexts. First, as *in vitro* correlates of *in vivo* genetic blocks to virus replication in specific

cells of the vaccinated host or the insect vector. Second, to identify a cell line suitable for production of vaccine stocks.

Growth of molecularly cloned mutants in neuronal cell lines

The growth of molecularly cloned attenuated strains of VEE was compared in different neuronal cell lines, Neuro2a, derived from a mouse neuroblastoma, and OBL21, a transformed line derived from mouse olfactory bulb tissue, as well as in primary dorsal root ganglia (DRG) cultured from rat embryo (Table 11). Several attenuated mutants, even those that showed little or no mortality following intracranial inoculation, were able to grow in both OBL21 and Neuro2a cells. However, for mutants giving little or no mortality in adult mice from a sc. footpad inoculation, the peak titers on OBL21 were more than a log lower than the titers on BHK cells. Included in this group was V3526, containing a deletion of the PE2 cleavage signal in conjunction with the E1 ser 253 resuscitator. V3526 also showed more than a log reduction of growth in Neuro2a cells. These cell lines provide a method to characterize neuroattenuation at the molecular level, and to identify mutations which may affect different steps in virus replication in these cells. The combination of these mutations in a single virus genome would decrease the likelihood of reversion to a more neurovirulent phenotype.

It is now possible to maintain DRG cultures for one month, and to greatly enrich these cultures for neurons (Levison and McCarthy, 1989). When DRG cultures prepared at the same time were infected with V3000 and several attenuated mutants, all but one of the viruses showed productive growth. V3014, a double mutant carrying E2 lys 209 and E1 thr 272, did not replicate, even though it is lethal when inoculated ic. Confirmation of this result will require further experiments with cultured DRGs.

TABLE 11
Growth of Molecularly Cloned Viruses in Neuronal Cells

Clone	Genotype	Percent Mortality		Peak titers (PFU/ml)[b]		
		footpad	ic.[a]	Neuro2a	OBL21	DRG
V3000	parent	100	100	$1x10^8$	$4x10^7$	$2x10^6$
V3526	E3 del 56-59 E1 253 phe>ser	0	14	$2x10^6$	$7x10^5$	$8x10^4$
V3040	E1 253 phe>ser	12.5	12.5	$1x10^8$	ND[c]	$1x10^6$
V3034	E1 272 ala>thr	62.5	75	$9x10^7$	$2x10^7$	$6x10^4$
V3010	E2 76 glu>lys	0	12.5	$7x10^8$	$4x10^7$	$4x10^6$
V3533	E2 76 glu>lys E2 116 lys>glu	0	87.5	$7x10^7$	$5x10^7$	$1x10^7$
V3032	E2 209 glu>lys	12.5	100	$1x10^{10}$	$8x10^7$	$6x10^7$
V3014	E2 209 glu>lys E2 239 ile>asn E1 272 ala>thr	0	87.5	$3x10^9$	$6x10^7$	<250

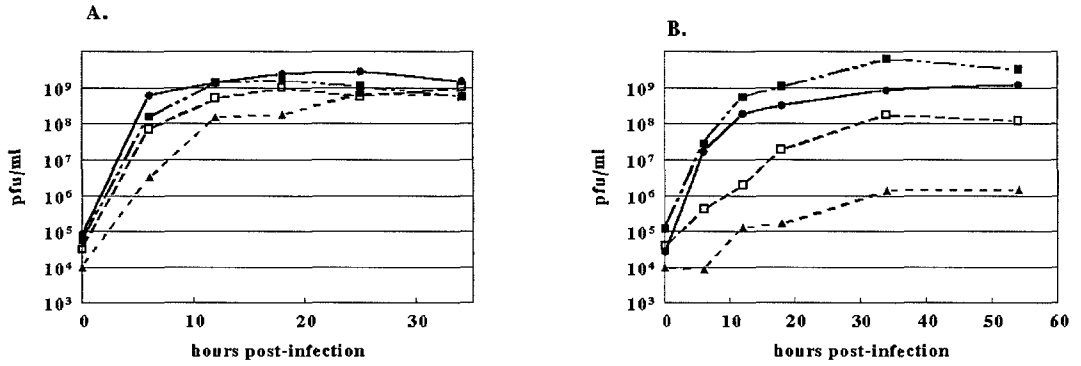
[a]intracranial inoculation; [b]peak cumulative titers for neuronal cells inoculated at ≥ 1 pfu/cell; [c] not done.

Growth in cultured mosquito cells

PE2-containing viruses derived from Sindbis strains AR339 and S.A.AR86 show restricted growth in mosquito cell lines (Presley et al., 1991; Heidner et al., 1994). PE2-containing VEE viruses also showed reduced growth in mosquitoes (See "Mutations Affecting Vector Infection and Transmission") and in C6/36 mosquito cells compared to BHK cells (Fig. 5). In this experiment, V3526, V3528 and V3040 (Table 3) were indistinguishable from V3000 with respect to growth on BHK cells, reaching titers of 10^9 pfu/ml within 24 hours after infection. However, on C6/36 cells, the two resuscitated mutants, V3526 and V3528, grew to lower titers than V3000 and V3040, both of which reached 10^9 pfu/ml or greater by 34 hours post-infection . V3526, with a deleted PE2 cleavage signal, gave the lowest titer on C6/36 cells, only 10^6 pfu/ml by 34 hr pi., while V3528, with an asp at E3 59, reached a titer of 10^8 pfu/ml. The correlation of reduced growth in C6/36 cells with growth in mosquitoes suggests that growth in C6/36 cells

might be used to screen other mutations for their effect on growth in the mosquito vector. This is a desirable phenotype which would reduce spread of a recombinant virus as well as decrease the opportunity for reversion during persistent infection of the mosquito.

FIGURE 5



Growth in Primary Mouse Embryo Fibroblasts

Fibroblasts cultured from mouse embryos (MEFs) represent an *in vitro* correlate of possible target cells in the rodent host. Analysis of infected MEFs may distinguish attenuated mutants blocked at different steps in replication. Also, in contrast to BHK cells, MEFs can produce and respond to interferons α,β, allowing study of the interaction of various mutants with this component of the innate immune system. Work with one of the molecularly cloned attenuated mutants, V3043, represents an example of this approach. A single nucleotide change from G to A at position 3 of the 5' noncoding region in mutant V3043 appears to alter the secondary structure of the genome RNA at its 5' terminus (J.-G. Wang and R. E. Johnston, unpublished results). This mutation, which was first identified in TC-83, leads to more rapid

clearance of the virus from the serum following footpad inoculation and from the brain following ic. inoculation (J.-G. Wang and R. E. Johnston, unpublished results). The timing of this clearance, beginning within 12 hours of infection, suggests that a nonspecific immune response, such as interferon, may be involved. *In vitro,* MEFs infected at low multiplicity of infection (m.o.i.) produce less V3043 than V3000, and less exogenous interferon is required to give a 50% reduction in the yield of V3043 and the cytopathic effect (CPE) induced by V3043 (Table 12). These phenotypes are also seen with the attenuated mutant, V3036, which contains a C substitution at nt 3, but not with V3045, a virulent mutant containing a U substitution at nt 3 (Table 12).

V3043 synthesizes greater amounts of the nonstructural replicase component, nsP1, both *in vitro* and in BHK cells (Table 13), possibly due to the altered structure of the 5' end of the genome RNA. Preliminary results suggest that the mutant produces more full-length positive and negative sense RNA in infected BHK cells. It may be the presence of elevated levels of double stranded RNA in the mutant infected cells that increases the effectiveness of interferon inhibition. There may be another factor in the attenuation of this mutant, however, because even MEFs infected at a higher multiplicity, where interferon would not be expected to have a large effect, produce significantly less mutant virus than parental virus (Table 12). This suggests an innate resistance of some mouse cell types to infection with the mutant virus. These characteristics may make this mutation a desirable component of a live virus vaccine for VEE.

TABLE 12
Growth of Molecularly Cloned Attenuated Mutants and Sensitivity to Interferon in MEFs

Clone	Growth on MEFs - Peak Titers		Sensitivity to Interferon - Units/ml required for:	
	Low m.o.i.[a]	High m.o.i.[b]	50% reduction of CPE	50% reduction of virus titer
V3000	6×10^7	1.25×10^7	3.2	3.2
V3043 - nt3 A	1×10^6	9×10^5	0.4	0.8
V3036 - nt3 C	9×10^6	8×10^5	0.4	0.8
V3045 - nt3 U	3×10^7	1×10^7	3.2	3.2

[a]50 pfu were used to infect approximately 2×10^7 MEF cells. [b]5 pfu/cell.

TABLE 13
Production of nsP1 *In vitro* and in infected BHK cells

Clone	*In vitro* translation[a]		In BHK cells[b]
	Rabbit Reticulocyte Lysates	Wheat Germ Extracts	
V3000	1.0	1.0	1.0
V3043	3.79	2.88	2.015
V3036	ND	ND	0.53
V3045	ND	ND	1.09

[a]Viral protein synthesis was programmed with run-off *in vitro* transcripts containing the first 1600 nucleotides of the viral genome. Viral proteins labelled with ^{35}S-methionine were immunoprecipitated with a polyclonal rabbit anti-nsP1 antibody, resolved by PAGE and quantitated by phosphoimaging. The value for V3000 was set at 1.0. [b]BHK cells were infected at an m.o.i. of 2, and proteins were labelled and quantitated as for *in vitro* translation.

Growth of V3526 in cultured cells - Identification of a cell line for vaccine production

1. BHK cells. Our previous studies with V3526 showed that variants with different plaque morphologies were produced during replication in BHK cells (Davis et al., 1995). Three plaque isolates of V3526 grown on BHK cells, one large, one small and round, and one small and irregular (like the V3526 parent) have been tested for virulence in CD-1 mice and for growth on BHK cells. CD-1 mice were inoculated with 10^3 pfu of each plaque variant sc. in

the left rear footpad (four mice per variant). No deaths or clinical disease resulted during the 14 day observation period, and all mice were protected against ip. challenge with 10^4 pfu of virulent V3000. These variants appeared during growth on BHK cells, suggesting that they may enjoy a selective advantage in these cells. However, one further cycle of growth on BHK cells gave low titers (approximately 5 x 10^5 pfu/ml) for all three variants. Therefore, although plaque variants did arise from growth of V3526 on BHK cells, the results of this experiment do not indicate that the variants have increased virulence in CD-1 mice, or improved growth properties in BHK cells.

It was observed in previous studies with V3526 and with other molecularly cloned attenuated mutants, that serial passage in BHK cells led to progressively higher maximum virus titers. We have done a careful passage study of V3526 in BHK cells to document how many cycles of infection are required for this effect, and to form the basis for experiments designed to determine its cause. The starting material was a V3526 electroporation supernatant from BHK cells containing 1 x 10^7 pfu/ml with a uniform small plaque morphology. BHK cell monolayers were infected at the indicated m.o.i. and supernatants were harvested at 24 hours. Titers were determined by plaque assay on BHK cells (Table 14).

TABLE 14
Serial Passage of V3526 in BHK Cells

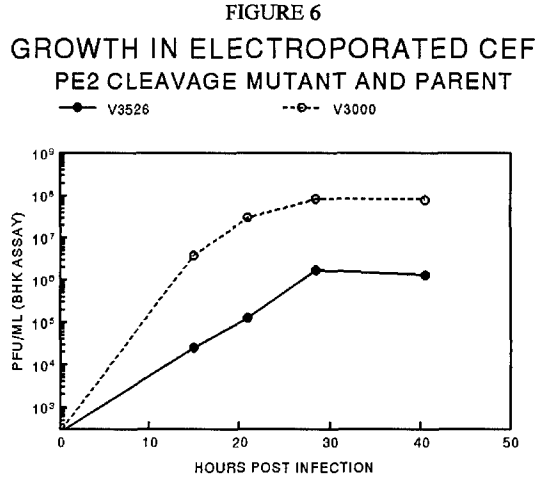
Sample	m.o.i.	virus titer (pfu/ml)	plaque size
electroporation supernatant		1.0 x 10^7	uniform, 1/4xnormal size
P1	0.35	1.6 x 10^7	10% 1/2xnormal, 90% 1/4xnormal
P2	2	2.2 x 10^8	10% 1/2xnormal to normal, 90% 1/4normal
P3	6	3.4 x 10^8	10% 1/2xnormal, 90% 1/4 normal

Because of the necessarily low m.o.i. in the first passage, this passage probably represents two cycles of infection. Therefore, in this experiment, an abrupt 10-fold increase in viral titer occurred between the second and third round of infection in BHK cells. Although large plaque variants did appear in the first low m.o.i. pass, they were not amplified in subsequent passes, and they were generally not as large as V3000 plaques ("normal size"). One possibility is that a mutant with a coding change in a viral protein was selected that makes either a more efficient RNA replicase/transcriptase, or an altered structural protein that is more efficient in attachment, penetration, uncoating, assembly and/or budding. Sequence analysis of virus produced by serial passage in BHK cells would determine whether this has occurred. Alternatively, a virus variant may have been selected with a poly(A) tract longer than the 21 residues in the pV3526 clone, which may improve either the efficiency of viral RNA replication or translation, or the stability of intracellular progeny genomes. There is a precedent for this effect in the case of EMC virus, a picornavirus. Stocks of EMC were divided by oligo(dT) chromatography into three subpopulations with average poly(A) tract lengths of 16, 26 and 74 residues. RNAs with the longest poly(A) tracts showed a 10-fold higher specific infectivity in transfection experiments than those with the shortest tracts (Hruby and Roberts, 1976). As poly(A) tracts in alphavirus virion RNA are of variable lengths (Johnston and Bose, 1972), the replicase is believed to stutter as it copies this sequence. If such stuttering were to occur on transfected *in vitro* transcripts, and a longer poly(A) tract gave a replicative advantage, selection of a variant with this attribute might occur. Analysis of the specific infectivity of viral RNAs isolated from different passages and correlation with length of poly(A) tracts would demonstrate whether this is in fact the case.

2. Chicken embryo fibroblasts. The fact that the biological homolog of V3526 (J9-1) was first isolated on primary chicken embryo fibroblasts (CEFs), coupled with the observation that electroporation stocks of V3526 made with BHK cells consistently contained only 1 x 10^7 pfu/ml, raised the possibility that V3526 was adapted to growth on CEFs and grew relative poorly in BHK cells. To determine whether CEFs are more permissive than BHK cells for V3526, and would give higher titer stocks with less selective pressure for variation, CEFs were transfected by electroporation with RNA transcripts from pV3526. Transfection supernatants produced with two different voltages both contained viable progeny virus. The plaques given by the supernatants from electroporated cells were small and uniform, but the titers were approximately the same or slightly less than those obtained from transfected BHK cells, about 1-5 x 10^6 pfu/ml for both conditions. These results suggested that CEFs may not be a more permissive cell type for this mutant.

Two further experiments were done to determine the ability of CEFs to replicate V3526. CEFs were transfected by electroporation with RNA transcripts of either pV3000 or pV3526 and virus growth was assayed over time (Fig. 6). V3526 grew more slowly than V3000 and the peak titer of V3526 was 100-fold lower than V3000. In a second experiment, parallel cultures of BHK and CEF cells were infected with V3000 and V3526 at an m.o.i. of 1 pfu/cell and virus growth was assayed. By 20 hr post infection of BHK cells, V3526 showed a titer of 1 x 10^8 pfu/ml compared to 7.8 x 10^8

FIGURE 6



pfu/ ml for V3000. CEFs, however, gave lower titers of both V3000 and V3526, 2.2 x 10^8 and 2.2 x 10^6 pfu/ml, respectively. Therefore, it does not appear that the resuscitating mutation, E1 ser 253, is specific for growth in CEF, and may in fact give more efficient growth in BHK cells than in CEFs.

3. Vero (African green monkey kidney) cells. In light of the results with CEFs, vero cells were chosen as a reasonable alternative for preparation of vaccine seed stocks. To adapt an attenuated PE2 noncleaving mutant to growth in vero cells, they were transfected by electroporation with nonviable RNA transcripts from clone pV3022. The transfected cells were distributed among the wells of a 96 well plate at varying densities and monitored for cytopathic effect. Between 108 and 132 hrs post transfection, 8 wells showed significant CPE. These supernatants were harvested and amplified by one round of growth on fresh vero cells. The BHK cell plaque morphology of each of these putative resuscitated noncleaving mutants was homogeneous and differed among the mutants and from the parent V3000. Five isolates were tested for virulence in six to nine week old female CD-1 mice by injecting 10^3 pfu into the left rear footpad. All were avirulent in this animal model, giving 0% mortality and no signs of illness. All of the isolates produced protective immunity against a challenge with 10^4 pfu of virulent V3000. When these isolates were tested for growth on vero cells, none of them grew to higher titers than did V3526 (0.2 to 1.5 x 10^7 pfu/ml), suggesting that it may not be possible to select a Vero cell specific resuscitating mutation that gives better growth than E1 ser 253. It is possible that these titers would not be high enough to produce an effective vaccine dose for humans with an acceptable amount of monkey cell DNA contamination.

4. MRC-5 (human diploid lung) cells. The growth of V3526 in MRC-5 cells was

assessed because a vaccine strain produced in these human cells, even if higher titers could not be obtained, would have a higher acceptable limit of DNA contamination and therefore would allow a larger volume per dose. Electroporation of MRC-5 cells with *in vitro* RNA transcripts of V3000 or V3526 reproducibly gave lower virus yields than those given by BHK cells, 90% lower for V3000 and 97% lower for V3526. As the production of a vaccine lot would require two passes of the vaccine strain in these cells, we infected fresh MRC-5 cells at two different m.o.i.'s, 0.05 and 1 pfu/cell, with the virus produced in this RNA transfection. V3526 titer (assayed on BHK cells) was about a log lower than V3000, and contained 43 to 78% large plaque variants. A second pass of these supernatants on fresh MRC-5 cells gave about the same final virus titer and no higher percentage of plaque size variants, suggesting that these variants did not have a selective advantage.

Ten plaques were picked from the first pass on MRC-5 cells, amplified on BHK cells and titered on BHK cells. All of the biologically cloned virus stocks were high titer (from 5 x 10^8 to 2 x 10^{10}) and varied in plaque size. MRC-5 cells were infected with two of the plaque isolates, M1 and M4, as well as the parental V3526 and V3000. M1 gave 1.4 x 10^7 pfu/ml at 48 hrs (complete CPE), with a plaque size smaller than V3000 but slightly bigger and rounder than V3526; M4 gave 1 x 10^8 pfu/ml with a plaque size equal to V3000, and V3526 grew to 4.8 x 10^6 pfu/ml. Four additional viruses were biologically cloned by plaque isolation from the second MRC-5 cell passage. In this case, an MRC-5 cell plaque assay and amplification on MRC-5 cells were used to avoid possible BHK cell-specific changes. When the growth of the four MRC-5 plaqued viruses on MRC-5 cells was examined, their peak titers did not differ significantly from that of the parental V3526. Therefore, the plaque isolates picked from and amplified on BHK

cells, but not those picked from and amplified on MRC-5 cells, showed improved growth on MRC-5 cells.

The uncloned electroporation stock of V3526, the MRC-5 passaged virus stocks and the plaque isolates were tested for virulence in mice by intracerebral (ic.) inoculation of 10^3 pfu into 6 week old and 2 week old CD-1 mice. None of these virus stocks caused illness or death in adult CD-1 mice, and the mice were completely protected against subsequent challenge (ip.) with 10^4 pfu of virulent V3000. Intracerebral inoculation of 2 week old mice with the V3526 electroporation supernatant virus killed 1/11 mice at 8 days with characteristic symptoms of VEE infection, while the V3526 stock passed twice in MRC-5 cells caused 2/12 deaths in these young mice. Therefore, two passages in these cells does not appear to select for more virulent viruses. However, plaque isolate M4 killed 12 of 14 two week old mice. M1 was not tested. This very stringent virulence test suggests that M4 is less attenuated than V3526. However, this isolate was harmless in adult mice. Further testing is needed using different routes of inoculation in adult mice to assess the attenuation and immunogenicity of M4. A panel of such isolates could form the basis for identification of MRC-5 adaptive mutations which could be used to improve the growth characteristics of V3526 in a certified human cell line without affecting its attenuation.

CONCLUSIONS

The tangible result of the work described in this report is the candidate vaccine strain, V3526, a molecularly cloned attenuated virus with the following properties. The clone

from which V3526 is derived contains a deletion of the four-amino acid PE2 cleavage signal that is lethal for viral replication, in conjunction with a resuscitating, and independently attenuating, change in the E1 glycoprotein (E1 ser 253). Otherwise, to the extent that the sequence of pV3526 is known, no other changes exist between this mutant and its parent, TRD. Therefore, V3526 virions contain an unprocessed PE2 surface glycoprotein rather than the mature E2 glycoprotein, but show the same specific infectivity for BHK cells and remarkable heat stability of the virulent parent. V3526 was avirulent in adult mice inoculated ip., sc., and in. and when inoculated directly into the brain was lethal for 1/14 mice at a dose of 10^3 pfu, and 1/6 mice at a dose of 10^5 pfu. Immunization by all routes gave protective immunity against a high dose ip. challenge with the virulent parent. Also, sc. or in. immunization with V3526 induced solid protection against intranasal or aerosol challenge with virulent VEE. Studies in mice indicated that V3526 introduced sc. in the footpad rapidly invaded and replicated in the draining lymph node and several other lymph nodes, including Peyer's patches, but unlike its parent, spared other organs and did not enter the central nervous system. This truncated pathogenesis profile may be the basis for the avirulence and immunogenicity of V3526. V3526 and other PE2-containing mutants showed restricted growth in the mosquito vector following intrathoracic inoculation. V3526 grew to acceptable titers in three cell lines used for production of vaccine stocks.

The V3526 mutant was the product of targeted mutagenesis of a region conserved among alphaviruses, the PE2 cleavage signal, followed by selection of second-site resuscitating mutations during growth in BHK cells. Analogous mutants of Sindbis virus (Heidner et al., 1994), and Semliki Forest virus (Glasgow et al., 1991) also have been described. Therefore, it appears likely that attenuated mutants such at V3526 could be generated for other alphavirus

pathogens, and also for other viruses that use the same glycoprotein processing pathway. This strategy is being actively pursued for EEE and WEE, as well as other serotypes of VEE by collaborators at USAMRIID. With this approach, the goal of using VEE as a model system is being realized.

The glycoprotein genes of V3526 have been completely sequenced. However, as one RNA transfection and two cell culture passages will be required to generate a vaccine stock, it is possible that innocuous nucleotide changes may occur during that process. Therefore, it was determined that the final sequence analysis would be deferred until the vaccine stock was produced.

In the process of identifying and characterizing V3526, useful tools for mutant analysis were developed, such as differentiated cell culture screens for host range mutants, a sensitive assay for reversion to a virulent phenotype in the infected animal, and quantitative assays for sensitivity to interferon and for the synthesis of the replicase component, nsP1. The application of these methods led to the following findings. The 5'-noncoding region was a second conserved region shown to contain an attenuating locus. A change at nt 3 from G to A is one of the attenuating mutations in TC-83 (Kinney at al., 1993). A substitution at nt 5 of Sindbis virus affects its virulence for neonatal mice (McKnight et al., 1996). An extensive study of the three possible changes at nt3 of VEE has shown that a C substitution is also attenuating. The nt3 A mutation results in a virus that is attenuated in mice when inoculated either sc. or ic., due to rapid clearance beginning as soon as 12 hours post-inoculation. This phenotype has been linked to increased sensitivity to interferon, increased production of the replicase component, nsP1, and perhaps to increased synthesis of viral RNA. These attributes may make the nt3 A or nt3 C

change an important constituent of a live virus vaccine for VEE.

The putative fusogenic region of E1, another conserved region, was a mutagenesis target. The results supported the strict requirement for a specific amino acid sequence in this region, as only one amino acid change of 17 tested gave a viable RNA genome. However, that mutant, E1 ile 81, was attenuated, as was a substitution at E1 83 in conjunction with an unmapped second site resuscitator. Therefore, it appears feasible to probe this region to identify additional mutations, should they be required.

Several triple mutant strains constructed and tested for virulence in rodents were shown to be safe and immunogenic. For some of these mutants, results suggested that they may not confer complete protection against virulent virus challenge under some conditions. It was clear, however, that each combination had a unique phenotype that was not predicted by the phenotypes of the constituent mutations.

Results of rodent studies presented here, and tests in horses and monkeys carried out by collaborators at USAMRIID, confirm the usefulness of this rational approach to vaccine design. The molecularly cloned V3526 vaccine candidate may prove to be the improved live virus vaccine for VEE, and the first of many genetically engineered vaccine strains.

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